Chloramine Mutagenesis in *Bacillus subtilis*

Abstract. *Chloramine (which occurs widely as a by-product of sanitary chlorination of water supplies) is shown to be a weak mutagen, when reversion of* trpC *to* trp$^+$ *in* Bacillus subtilis *is used as an assay. Some DNA-repair mutants appear to be more sensitive to chloramine, suggesting the involvement of DNA targets in bactericide. The influence of plating media on survival of cells treated with chloramine suggests a bacterial repair system acting upon potentially lethal lesions induced by chloramine.*

Chloramine (NH_2Cl), the reaction product of chlorine and ammonia in aqueous solution, is widely used directly for, or is generated as a by-product of, the disinfection of public water supplies and swimming pools. Nevertheless, little is known of the biological mechanism of action of chlorine or of possible genetic effects. Our own study (*1*) has shown that chloramine reacted with *Bacillus subtilis* DNA in vivo and in vitro; however, Boyle (*2*) did not obtain auxotrophs by treating *Escherichia coli* cells with chloramine; and a preliminary experiment with bacteriophage lambda by Hayatsu (*3*) did not show mutagenicity of hypochlorite. On the other hand, while this manuscript was in preparation, Wlodkowski and Rosenkranz (*4*) reported that sodium hypochlorite was a weak base-substitution mutagen in *Salmonella typhimurium*. We now report the mutagenicity of chloramine, using reversion of *trp*C to *trp*$^+$ in *B. subtilis* as an assay.

The strains used in this study were derivatives of indole-requiring *B. subtilis* strain 168 (*5*). As an index of the involvement of DNA targets in bactericide by chloramine, the sensitivity of different *B. subtilis* strains carrying various DNA-repair mutations was examined. Cell concentrations of each mutant and of its control for chloramine treatment were adjusted to about the same. In addition, the mixed cultures of strains 168 + SB879 and strains BD170 + BD194 were treated with chloramine and their survivors were sorted out by their respective nutritional markers. The surviving fractions of a representative test for each mutant as a function of chloramine doses are shown in Fig. 1. While *uvr* (SB879) and *rec*B (BD191) showed no evident increases in sensitivity, *rec*3 (BD193), *rec*A (BD194), and *pol*A5 (SB1060) were consistently more sensitive than their respective controls. Another test of the *pol*A5 mutant in a different strain, SB1059 (*pol*A5, *phe*A, *trp*C), demonstrated the same chloramine sensitivity as SB1060 did. [This finding is consistent with that obtained by Rosenkranz (*6*) which was also reported in the course of these experiments.] Our data suggested that at least *pol*A5$^+$, and possibly *rec*A$^+$ and *rec*3$^+$, are involved in the repair of chloramine-induced damage in DNA.

Strain 168 (*trp*C) was used to test the influence of plating media on survival after chloramine treatment and the effect of chloramine on mutation. Ultraviolet irradiation was employed to give comparative results. In ultraviolet-irradiated cells, the survival on nutrient agar was much lower than that observed on all the other media (Table 1). Minimal medium, however, showed the lowest survival after chloramine treatment. Since chloramine readily reacted with amino acids and proteins (*2*), the supplementation of nutrients after chloramine treatment seemed to be essential in the recovery of the cells. Among the nutrient-supplemented media, however, amino acid supplements (AA, CH-trp) afforded more efficient repair than nutrient agar for both ultraviolet-irradiated and chloramine-treated cells. The tests for the prototrophs (SB19 and SB850, *trp*$^+$) gave similar results. This phenomenon was similar to that observed in ultraviolet irradiation of *E. coli* (*7*), and it was suggested that the higher survival on synthetic medium than on complex medium resulted from delaying the growth of the irradiated cells until repair of ultraviolet damage occurred. The differential recoveries of chloramine-treated cells also suggested a bacterial repair system acting upon potentially lethal lesions, similar to

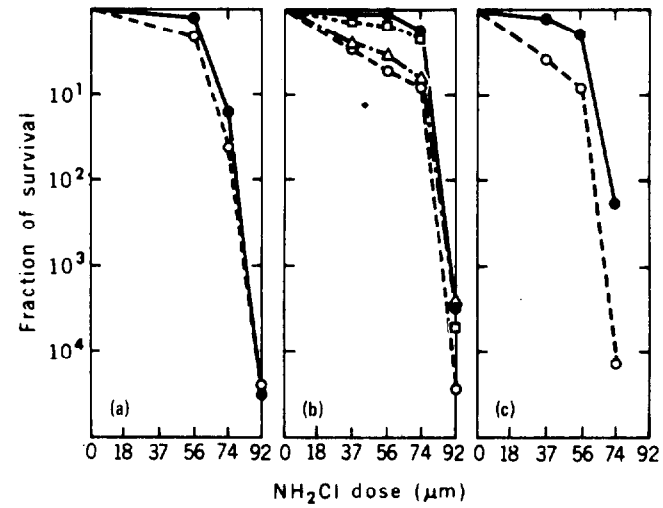

Fig. 1. Chloramine sensitivity of different *B. subtilis* strains. (a) ●, 168 (*uvr*$^+$, *trp*C); ○, SB879 (*uvr*, *trp*C, *his*B); (b) ●, BD170 (*rec*$^+$, *trp*C, *threo*5); □, BD191 (*rec*B, *trp*C, *threo*5); △, BD193 (*rec*3, *trp*C, *threo*5); ○, BD194 (*rec*A, *trp*C); (c) ●, SB1058 (*pol*$^+$, *phe*A, *trp*C, *his*B); ○, SB1060 (*pol*A5, *phe*A, *trp*C, *his*B). For the chloramine treatment, NaOCl was diluted in 0.05*M* phosphate buffer, *p*H 7, to different concentrations. Chloramine (NH_2Cl) was formed by incubating eight parts of NaOCl and one part of 0.1*M* NH_4Cl at 37°C for 1 hour. The prepared early stationary phase cells (*1*) were diluted ten times into chloramine solution and treated for 30 minutes at 37°C. The reaction was stopped by the addition of one volume of 0.02*M* sodium thiosulfate. Viable cell counts were scored by averaging the colonies of two plates (amino acid media, see Table 1) after incubation at 37°C for 2 days. The minimum number of colonies per plate was 161 ± 8 percent.

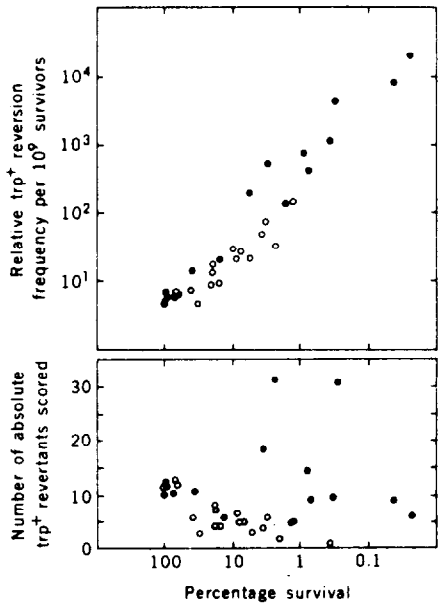

Fig. 2. Relative *trp*$^+$ reversion frequencies and absolute *trp*$^+$ revertants plotted against the percentage of survival after chloramine treatment (●) and ultraviolet irradiation (○). The revertants and survival were scored on CH and CH+trp media, respectively. Cell counts on CH+trp medium were 2.26×10^9 to 2.61×10^9 for controls.

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Table 1. Effects of different media on survival and on *trp*C to *trp*$^+$ reversion after treatment with chloramine or ultraviolet irradiation. The media used were as follows: NA, nutrient agar (Difco); MM, Spizizen minimal medium + 0.5 percent sucrose + 1.75 percent agar; MM+trp, MM + tryptophan (25 μg/ml); CH, MM + 4 mg of casein hydrolyzate per milliliter (Nutritional Biochemicals); CH+trp, CH + tryptophan (25 μg/ml); AA−trp, MM plus all the amino acids (25 μg/ml) except tryptophan; AA, MM plus all the amino acids (25 μg/ml). Viable cells were counted as described in Fig. 1. The revertants were counted after 4 days of incubation at 37°C.

Treatment	Survival (%)				*trp*$^+$ reversion*		
	NA	MM+trp	CH+trp	AA	MM	CH	AA−trp
NH_2Cl							
None	95.11	95.74	100†	100.53	11 (5.1)	8 (3.5)	6 (2.6)
18 μM	78.19	70.29	98.94	90.43	10 (5.3)	9 (4.0)	11 (5.4)
37 μM	39.88	28.09	56.25	64.19	4 (6.3)	14 (11.1)	11 (7.6)
56 μM	0.69	0.25	2.47	13.67	2 (315)	32 (574)	24 (78)
74 μM	0.0037	0.0013	0.042	0.27	0 (−)	8 (8547)	6 (983)
Ultraviolet							
None	101.15	100.38	100†	98.08	10 (5.1)	12 (4.6)	13 (4.7)
1 min	23.53	71.33	65.89	65.87	8 (3.9)	13 (7.7)	12 (7.6)
3 min	4.70	17.59	15.63	20.65	4 (8.7)	4 (9.8)	9 (15.2)
6 min	0.30	2.76	2.10	5.13	3 (41.6)	2 (36.4)	3 (22.3)
9 min	0.0068	0.38	0.38	1.00	1 (−)	1 (−)	2 (762)

*Upper numbers are actual numbers of revertants scored; lower numbers in parentheses are relative frequencies per 10^9 survivors. For ultraviolet treatment the prepared cells were suspended in phosphate buffer (3.5 ml) and held at 37°C for 30 minutes; they were then treated with ultraviolet (5 erg sec^{-1} mm^{-2}) in plastic petri dishes (5 cm in diameter). †Cell counts are 2.26×10^9 for NH_2Cl treatment and 2.61×10^9 for ultraviolet irradiation.

well-known findings with ultraviolet irradiation.

The results of two typical experiments on the effect of chloramine and ultraviolet irradiation on the yield of *trp*C to *trp*$^+$ reversion are shown in Table 1. In ultraviolet-irradiated cells plated on different media and in chloramine-treated cells plated on minimal medium, there was enhancement of mutation frequencies; however, the absolute number of revertants was reduced owing to the superimposed lethality. The use of supplemented media for the chloramine-treated cells, in contrast, yielded small but consistent increases of total numbers of *trp*$^+$ revertants (and implies marked enhancement of *trp*$^+$ reversion rates). This result resembled that observed in *S. typhimurium* and *E. coli* (*8*): that the yield of *trp*$^-$ to *trp*$^+$ reversion induced by ultraviolet irradiation was profoundly enhanced by certain conditions of incubation after treatment.

The *trp*C marker of *B. subtilis* is a stable locus with a low spontaneous reversion frequency of 2 to 9 per 10^9 cells. Absolute increase of revertants was observed only at survivals between 0.1 and 10 percent. However, chloramine kill was difficult to control because of the presence of other variables (such as cell concentration, amino acid or protein residues in cell samples, and the cleanliness of glassware) and the same dose of chloramine did not always give reproducible kill and reversion. These small increases in absolute numbers of *trp*$^+$ revertants reinforced the evidence for mutagenesis by chloramine, as judged by the relative increases among the survivors. The combined results of several experiments were analyzed statistically (Fig. 2). Comparing the regression coefficiencies (relative reversion frequencies and percentage of survivals) of the two killing agents, chloramine ($b = -43.62$) had a significantly ($d = 3.99$) higher effect, for a given kill, than ultraviolet did ($b = -0.66$), in enhancing the *trp*$^+$ reversion. The chloramine sensitivity of 12 *trp*$^+$ revertants was also studied, and they were as sensitive to chloramine as the *trp*C cells.

The effect of chloramine on the yield of *trp*C to *trp*$^+$ of other different strains carrying various mutation pertinent to DNA repair was also studied on CH medium. The preliminary experiments showed that the *trp*$^+$ yields of *pol*A5 (SB1060), *rec*3 (BD193), *rec*A (BD194), and *uvr* (SB879) were not significantly different from the yields of *pol*A5$^+$ *rec*$^+$ control strains (168, BD170) when treated with chloramine. Strain *rec*B (BD191), however, had a comparatively lower reversion frequency whether the cells were chlorinated or not. No increase of absolute number of revertants in chloramine-treated cells was seen. Similar phenomena have been reported also in studying the mutability of recombination-deficient and ultraviolet-sensitive strains of *E. coli* toward ultraviolet light (*9*).

The *trp*$^+$ revertants of 168 have been studied for the transforming activity of the linkage group (*aro*B$^+$ *trp*C$^+$ *his*B$^-$). Of 75 chloramine-induced revertants, 24 percent showed reduced *trp*$^+$ transforming activity compared to wild type *trp*$^+$. Spontaneous reversions showed about 8 percent such transforming-defective types. These may represent more complex genetic changes than base substitution.

KATHERINE LU SHIH*
JOSHUA LEDERBERG
Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

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* Present address: Biological Adaptation Branch, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, Calif. 94035.

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